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Synthesis, depletion and cell-type expression of a protein from the male accessory glands of the dengue vector mosquito *Aedes aegypti*

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ABSTRACT

Aedes aegypti males transfer sperm and seminal fluid proteins (Sfps), primarily produced by male accessory glands (AGs), to females during mating. When collectively injected or transplanted into females, AG tissues and/or seminal fluid homogenates have profound effects on *Aedes* female physiology and behavior. To identify targets and design new strategies for vector control, it is important to understand the biology of the AGs. Thus, we examined characteristics of AG secretion and development in *A. aegypti*, using the AG-specific seminal fluid protein, AAEL010824, as a marker. We showed that AAEL010824 is first detectable by 12 h post-eclosion, and increases in amount over the first 3 days of adult life. We then showed that the amount of AAEL0010824 in the AG decreases after mating, with each successive mating depleting it further; by 5 successive matings with no time for recovery, its levels are very low. AAEL010824 levels in a depleted male are replenished by 48 h post-mating. In addition to examining the level of AAEL010824 protein, we also characterized the expression of its gene. We did this by making a transgenic mosquito line that carries an Enhanced Green Fluorescence Protein (EGFP) fused to the AAEL0010824 promoter that we defined here. We showed that AAEL010824 is expressed in the anterior cells of the accessory glands, and that its RNA levels also respond to mating. In addition to further characterizing AAEL010824 expression, our results with the EGFP fusion provide a promoter for driving AG expression. By providing this information on the biology of an important male reproductive tissue and the production of one of its seminal proteins, our results lay the foundation for future work aimed at identifying novel targets for mosquito population control.

1. Introduction

The mosquito, *Aedes aegypti*, is an important vector of viruses that cause dengue and Chikungunya infections (Gubler, 2002). Dengue affects an estimated 390 million people worldwide each year, and the majority of the world's population is now at risk for infection (Bhatt et al., 2013). With no vaccine available and no treatment for dengue or Chikungunya, control of these diseases is focused on limiting the spread of, or transmission by, their mosquito vector (Vazquez-Prokopec, 2011). However, vector control has been difficult due to numerous factors, including expanding urbanization and the lack of effective mosquito control tools (Morrison et al., 2008). Accordingly, it is essential to develop novel tools for vector control that will ultimately reduce the burden of vector-borne infections.

Reproduction of the mosquito vector is a key process in the spread of mosquito-borne diseases, and thus a worthwhile target to consider for disease control. In numerous insect species, including *A. aegypti*, Sfps are transferred along with sperm from males to females during copulation. Sfp receipt in the mated female effects numerous physiological and behavioral changes (reviewed in Avila et al. (2011) and Gillott (2003)). These changes include stimulating oviposition (Judson, 1967), increasing fertility (Adlakha and Pillai, 1975), changing female host seeking and feeding behavior (Lee and Klowden, 1999), and increasing female mating refractoriness (Fuchs et al., 1968; Helinski and Harrington, 2011).

In insects, Sfps are typically produced in the male accessory glands (AGs), yet the structure and secretory activity of these glands have received little attention. Most AGs consist of a secretory epithelium surrounded by a muscle sheath. Secretory epithelia of the AG, like most insect epithelia, are monolayers, in

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which each secretory cell produces, stores and exports secreted products (Happ, 1984; Gligorov et al., 2013). *A. aegypti* AGs are divided into anterior and posterior zones that differ in cell type, density and in the nature of the secretory material that they contain (Dapples et al., 1974). Transplantation of cells from the anterior compartment of the AGs into unmated females inhibits multiple inseminations and stimulates oviposition in females (Ramalingam and Craig, 1976), whereas the cells of the posterior region synthesize and secrete a mucus that serves to bind the secretory granules produced in the anterior region (Ramalingam, 1983).

Development of the male *A. aegypti* AGs begins during the larval stage, but is not complete until 24 h post adult eclosion, when the lumens of the ejaculatory duct and AGs fill with secreted products (Clements, 1999). Several potential secretion mechanisms have been suggested to operate in the AGs of various insects. These mechanisms include merocrine (secretion via exocytosis), holocrine (secretion products are released by rupture of the cell membrane) and apocrine secretion (where the apical portion of the cell is released along with secretion products). Merocrine and perhaps holocrine secretion have been suggested for *Drosophila* AGs (Chen, 1984; Perotti, 1971) and the flour moth, *Anagasta* (Riemann and Thorson, 1976). Merocrine secretion has also been described for the AGs of the butterfly *Calpodes* (Lai-Fook, 1982) and the darkling beetle *Tenebrio*, and apocrine secretion has been reported in the AGs of the Colorado potato beetle (*Leptinotarsa decemlineata*) (reviewed in Happ (1984)).

In *A. aegypti*, a variety of secretory mechanisms have been suggested for the AGs. Jones (1970) observed numerous male AG-derived cellular organelles and fragments, including mitochondria and membranes, inside the female bursa, leading him to suggest a holocrine secretion. Subsequent electron microscopy studies by Dapples et al. (1974) suggested apocrine secretion by both anterior and posterior AG cells. Further studies attributed apocrine secretion to the AG anterior cells, and holocrine secretion in the AG posterior cells in both *A. aegypti* and *Aedes triseriatus* males (Ramalingam, 1983; Ramalingam and Craig, 1978; Sirot et al., 2011). Thus, there remains debate about the secretory mechanism used by *A. aegypti* AG cells.

Another debate concerns the effect of mating on synthesis of male AG secreted proteins. Similarly to the majority of male insects, female *Aedes* are primarily monogamous and male *Aedes* are polygynous (Clements, 1999); during each copulation, a proportion of the AGs' secreted material is transferred at ejaculation and, subsequently, must be replenished. Dapples et al. (1974) and Foster and Lea (1975) reported that males' depleted AGs regenerated and refilled over few days. However, Ramalingam (1983), Ramalingam and Craig (1976) and Hausermann and Nijhout (1975) argued that recovery of secretory capacity did not occur after depletion.

Elucidating male AGs function—the role of individual cell-types and their patterns of secretion—is a first step in dissecting the overall contribution of this organ to mosquito reproductive biology and might potentially identify targets for the purposes of reproductive control. Therefore, we conducted a comprehensive investigation of the male AGs. Previously, we identified 93 seminal proteins from *A. aegypti* (Sirot et al., 2011). We used one of these, the AG-specific protein AAEL010824 as a marker for the present studies. We characterized the expression of *AAEL010824*, examining both its transcript and protein levels during development, as well as before and after mating. We determined how quickly males that mated in rapid succession became depleted of AAEL010824, and how much time was required until the protein was replenished in these males. Finally, we used the upstream region of the *AAEL010824* gene to drive the reporter Enhanced Green Fluorescence Protein (EGFP) in transgenic mosquitoes. We showed that this regulatory region drives expression in anterior cells of AGs, specifically. We then examine protein transfer from the anterior cells of the AGs.

2. Methods

2.1. Mosquitoes

A. aegypti (Thai strain) were originally collected in Bangkok, Thailand (15°7193′N, 101°752′E) in 2011, and supplemented with field material in 2012. This colony was held in an environmental chamber at 25.9 ± 0.6 °C with 71.9 ± 9.5% relative humidity (RH), with a photoperiod of 10-h light:10-h dark with a 2 h simulated dusk and dawn period. Mosquitoes were reared to obtain uniform medium body size adults. Larvae were fed on Cichlid gold pellets (Hikari, Himeji, Japan) using four pellets per tray of 200 larvae. Adults had constant access to 10% sucrose. Individual pupae were transferred to vials to ensure virginity and sorted by sex upon adult eclosion. Two hundred individuals were transferred into 12 L plastic mating cages by sex and held until experiments commenced.

2.2. Mosquito matings

Matings were conducted as described previously (Helinski and Harrington, 2011). Five-day-old medium body size *A. aegypti* males and females were used in our experiments. One virgin male was released into a 5 L observation cage containing approximately 8 virgin females. Male and female couples were observed carefully and copulating pairs were removed using a mouth aspirator after a minimum mating duration of 10 s. For our multiple mating experiments, males were mated in succession to a different numbers of females (from 1 to 5) by transferring them to subsequent cages with virgin females following the process described above. Males from each mating frequency group (those males mated to 1, 2, 3, 4 or 5) plus virgin males were collected each day for 3 days. These males were frozen and stored at −80 °C for Western blot analysis or RNA extraction.

2.3. Rapid amplification of cDNA ends (RACE)

RACE was employed to determine the 5′ and 3′ UTR sequences and to validate the open reading frame (ORF) of *AAEL010824*. Total RNA from whole males was isolated using TRIzol solution (Invitrogen, Carlsbad, California), followed by chloroform/isopropanol extraction and ethanol precipitation according to standard protocols. Prior to cDNA synthesis, RNA was treated with RNase-free DNAse (Clontech, Madison, Wisconsin). RACE was performed using the GeneRacer system (Invitrogen. Carlsbad CA, USA) with SuperScript III (Invitrogen. Carlsbad CA, USA), following the manufacturer's instructions. Oligo dT primer (5′ GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA TGT $(T)_{24}$ 3′) was used to reverse transcribe the 3′ end of the mRNA. Primer 5′cDNAsynthGSP: 5′TCG GAG CCC CTT ATG TAG ACG TA 3′ and Random (N6) primers were used to reverse transcribe the 5′ end of the mRNA. The samples containing the cDNA for analysis of the 3′ and 5′ ends were diluted 10-fold, and 1 μL was used as PCR template. Primer GeneRacer 3′ (5′ GCT GTC AAC GAT ACG CTA CGT AAC G 3′) and 3′RACE-GSPF1 (5′ TGG CGA CAT GTG GGT CAT TAC CAG AA 3′) were used for 3′ ends PCR. Primer GeneRacer 5′ (5′ CGACTG GAG CAC GAG GAC ACT GA 3′) and 5′RACE-GPSR3 (5′ ACG CGA CTT TCG CAC GGA CA 3′) were used for 5′ends PCR. PCR products of 3′ and 5′ ends were cloned into pCR4-TOPO vector (Invitrogen, Carlsbad CA, USA) and transformed into TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad CA, USA). Plasmids were sequenced by the Cornell University Life Science Core Facility. Sequences were analyzed to identify the 3′ and 5′ UTR of the *AAEL010824* mRNA.

2.4. Gene analysis

Gene analyses were carried out using the Genious software package (Pro 5.6.5, Biomatters, Auckland, New Zealand). DNA

sequence alignments to the *A. aegypti* genome were performed with BLAST (www.vectorbase.org/blast), while multiple sequence alignment and phylogenetic analysis were done using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). SignalP 4.1. (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the putative signal peptide. Sequences from predicted *Aedes albopictus* proteins were obtained from Boes et al. (2014). Sequences from predicted *A. aegypti* proteins were obtained from Sirot et al. (2011) and Vectorbase.org.

2.5. AAEL010824 promoter-EGFP construct

In order to study *AAEL010824* expression and to characterize secretion by the AG cells, we created a construct using the promoter of *AAEL010824* to drive expression of EGFP. The NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview), Promoter 2.0 Prediction Server (CBS, Technical University of Denmark DTU, http://www.cbs.dtu.dk/services/Promoter/) and our 5′RACE data were used to estimate a predicted regulatory region of the *AAEL010824* gene. We BLASTed the sequence against the *A. aegypti* genome in Vectorbase (www.vectorbase.org) and amplified the sequence extending from the transcription start site determined in the 5′RACE to approximately ~5 kb upstream using iProof High Fidelity DNA Polymerase (Biorad, Hercules, CA,USA) following the manufacturer's protocol. Primers used to amplify this region were 10824(5K)-FseI-F: TAA TAG GCC GGC CCT GGG CTC GTT AAT CTC GAA and 10824(5K)-FseI-R: TAA TAG GCC GGC CGA ATA ACG GAT GTC ACA AGA ACT GGC TG. The final PCR product was cloned into pCR-XLTOPO (Invitrogen Carlsbad, CA, USA) and transferred to pBAC[3xP3-EGFP-DsRedaf] using the FseI sites of the final pBAC [3xP3-EGFP-DsRedaf] plasmid (Fig. 3A). To generate pBAC[3xP3-EGFP-DsRedaf], an EGFP-SV40 fragment was amplified from pBac[3xP3-EGFPafm] plasmid and cloned into the FseI-AscI sites of pBAC[3xP3-DsRedaf]. The EGFP fragment amplified does not include a secretion signal. The final construct was sent to the Insect Transformation Facility at the University of Maryland for injection into *A. aegypti* strain to create a transgenic line. The line was maintained by continuously crossing EGFP-positive females (verified by PCR) with EGFP-positive males.

2.6. RNA isolation and quantitative PCR analysis

To quantify *AAEL010824* transcript levels at all developmental stages, and before and after mating, total RNA from whole males was isolated using TRIzol solution (Invitrogen, Carlsbad, California), followed by chloroform/isopropanol extraction and ethanol precipitation according to standard protocols. Prior to cDNA synthesis, RNA was treated with RNase-free DNAse (Clontech, Madison, Wisconsin). Reverse transcription was conducted using 1ug of total RNA following the manufacturer's instructions (Clontech, Mountain View, CA). Relative transcript levels of *AAEL010824* were measured using quantitative PCR conducted with a CFV96 Real-Time System (Bio-Rad, USA). Two *AAEL010824* gene specific primers 5′GAT ATT GGG TCT TGT GTT AAG TGC3′ and 5′AGT TGC CGG TCG CTG TTC G3′ were used to amplify an 82 bp amplicon. Both *S17* and *Actin* (Cook et al., 2006) were used as controls. Primers 5′ACG ACA GCA GCG AAA CTT GAA TCA3′ and 5′ATC TTA TTG CGC AGG GGC TTC G3′ were employed to amplify 78 nucleotides of the *S17* gene. Primers 5′ATC GTA CGA ACT TCC CGA TG3′ and 5′GAA CGA TGG CTG GAA GAG AG3′ amplified 81 nucleotides of the *Actin* gene. Amplifications were carried out in a total volume of 15 μL containing 7.5 μL of iQ SYBR Green Supermix (Biorad, USA), 1 μL cDNA and 0.5 μL of each primer. Cycle differences between *AAEL010824* and either S17 or actin genes (ΔC_T) were compared to generate the relative expression of *AAEL010824* at different time points and life stages.

2.7. Western blotting

Western blotting was used to examine levels of AAEL010824 and EGFP proteins. Western blotting was carried out as in Ravi Ram and Wolfner (2009). Briefly, male reproductive tracts were dissected in 1× PBS under a dissecting microscope. Tissue was placed in 10 μL of 1× PBS in an Eppendorf tube, and ground briefly with a pestle. After adding 10 μL of 2× SDS sample buffer, samples were boiled for 5 min and centrifuged. The supernatant was loaded into a SDS 5–15% gradient polyacrylamide gel. After electrophoresis, the proteins were transferred onto Immobilon PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat dry milk blocking solution for 1 h, followed by probing using rabbit anti-AAEL010824 at a concentration of 1:1000, mouse anti-alpha-Tubulin (Sigma T5168, clone B-5-1-2) at 1:10,000 or rabbit anti-GFP (Life Technologies, Grand Island, NY) at 1:1000, overnight at 4 °C. Incubation with secondary goat anti-rabbit and goat anti-mouse was performed at 1:2000 (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Blots were treated with Pierce ECL 2 Substrate (Thermo Scientific Pierce, Logan, UT) and imaged using a Typhoon 8600 imager (GE Healthcare, Piscataway, NJ). Rabbit anti-AAEL010824 was raised against the synthetic peptide 173YVDDEKHDQRNAYN186 and affinity-purified (Genscript, NJ, USA). Band intensity was measured using Image studio lite software (Li-COR corporate, Lincoln, Nebraska, USA). *AAEL010824* signals normalized to tubulin loading control were compared to generate the relative AAEL010824 protein content at different mating groups during different time points.

2.8. Immunofluorescence and microscopy

We used confocal microscopy to determine the expression pattern driven by the regulatory region of *AAEL010824*. Specifically, we examined EGFP localization in transgenic mosquitoes that expressed the fluorescent protein under the control of the *AAEL010824* upstream regulatory region. Accessory glands were dissected in 1× PBS and fixed with 4% paraformaldehyde (Sigma, MO, USA) at room temperature for 30 min. Fixed tissue was washed, permeabilized in 0.2% Triton X-100 (Sigma, MO, USA) for 10 min, blocked for 30 min with bovine serum albumin (Sigma, MO, USA) and incubated with anti-EGFP (1:1000) at 4 °C overnight. Slides were washed and then incubated with secondary goat-anti-rabbit-Alexa 488 (Jackson Immunoresearch, PA, USA) for 1 h at room temperature (1:200). Accessory glands were then incubated with DAPI in PBS at 1ug/mL for 20 min at room temperature to stain DNA. Prolong gold antifade reagent (Invitrogen, Carlsbad, California) was used as mounting medium. For confocal microscopy, sequential excitation was performed at 488 nm (for fluorescein) and 405 nm (for DAPI) with a Zeiss 710 confocal microscope. The images were processed using Zen software (Zen 2011).

3. Results and discussion

3.1. Characterization of the AAEL010824 gene

Our previous work demonstrated that AAEL010824 is transferred to females during mating (Sirot et al., 2011). To examine the structure of the RNA encoding this protein, we used Rapid Amplification of cDNA ends (RACE). The *AAEL010824* transcript contains a 22 bp 5′-untranslated region (UTR) and a 624b coding sequence that is followed by either of two 3′-UTR variants. The shorter 3′-UTR variant is 48b in length, while the other variant is 726b. *AAEL010824* is predicted to encode a protein of 207 amino acids. A paralog of *AAEL010824*, *AaegSfp8*, was previously described in *A. aegypti* (Sirot et al., 2011). *AAEL010824* and *AaegSfp8* are located on the same contig, within 34 kb of each other. These

two sequences have 70% identity (*E* value = $2e^{-74}$) due primarily to variability in the N-terminal region of the predicted proteins. Potential signal peptide cleavage sites were detected in both amino acid sequences, indicating that both proteins might be secreted (Fig. 1A). Given the high similarity between these two proteins, we suggest that the genes could be the result of a tandem duplication of ancestral gene, followed by divergence. Such genesis of seminal protein-encoding genes has been documented previously in numerous cases in Drosophila (e.g. Clark et al., 2007; Wagstaff and Begun, 2007; Findlay et al., 2008; Sirot et al., 2014).

A BLAST database search revealed that the amino acid sequences of AAEL010824 or *AaegSfp8* are not similar to known proteins in other insect species, including proteins in *Anopheles* and *Culex* mosquitoes. However, there are three transferred *A. albopictus* Sfps that have 68% identity with AAEL010824 over the entire length of the protein (Boes et al., 2014). This finding is interesting as Leahy (1965) showed that implantation of *A. albopictus* male AGs into unmated *A. aegypti* females increased oviposition. In addition, Tripet et al. (2011) showed that injection of *A. albopictus* Sfp homogenates into unmated *A. aegypti* females induced female mating refractory behavior, suggesting that *A. albopictus* males have proteins that can trigger some post-mating responses in the *A. aegypti* females. To investigate the phylogenetic relationship of these proteins, we constructed a phylogenetic tree based on the amino acid sequences of the three *A. albopictus* genes, *AaegSfp8* and *AAEL010824*. Phylogenetic analysis using the Neighbor Joining method (Saitou and Nei, 1987) indicated that these sequences may share a common ancestor, where *AaegSfp8* and *A. albopictus* 16385 and 5389 are positioned in the same sub-clade that is located within a main clade along with *AAEL010824* and *A. albopictus* 8692 (Fig. 1B). These phylogenetic data provide support for a gene duplication event in which all the family members are proteins transferred from male to female during copulation; the divergence of these two groups could reflect differences in their post-copulatory functions after transfer.

3.2. AAEL010824 expression in pupae and adult males

Aedes mosquitoes' AGs begin development during the larval stage. They mature rapidly during the late pre-adult stages, reaching full secretory activity in young adults (Clements, 1999). Previous studies characterized the physical changes of *A. aegypti* AGs that follow accumulation of secretory material (Dapples et al., 1974; Foster and Lea, 1975). In those studies, investigators noted an increase in AGs diameter within 1 h after eclosion (interpreted as an indicator of seminal fluid accumulation in the AGs increasing their volume). This increase ceased after approximately 2 days. However, a direct relationship between AGs diameter, seminal fluid protein content and AGs activity was never established in those studies.

To characterize the expression of genes in the male AGs, we first examined transcript levels of *AAEL010824* across development, using quantitative PCR. Low mRNA levels were detected in the pupal stage. *AAEL010824* mRNA levels increased after eclosion for at least 3 days (Fig. 2A). We then examined *AAEL010824* protein levels, which we expected might be more directly related to AGs diameter. Using a purified AAEL010824 antibody raised against a synthetic peptide, we did not detect AAEL010824 in Western blots of proteins from pupae or unmated male adults at 3 h post-eclosion (Fig. 2B). However, AAEL010824 was detected in unmated male samples from 12 h post-eclosion to 3 days post-eclosion, with increasing levels seen for up to 2–3 days post-eclosion. These data

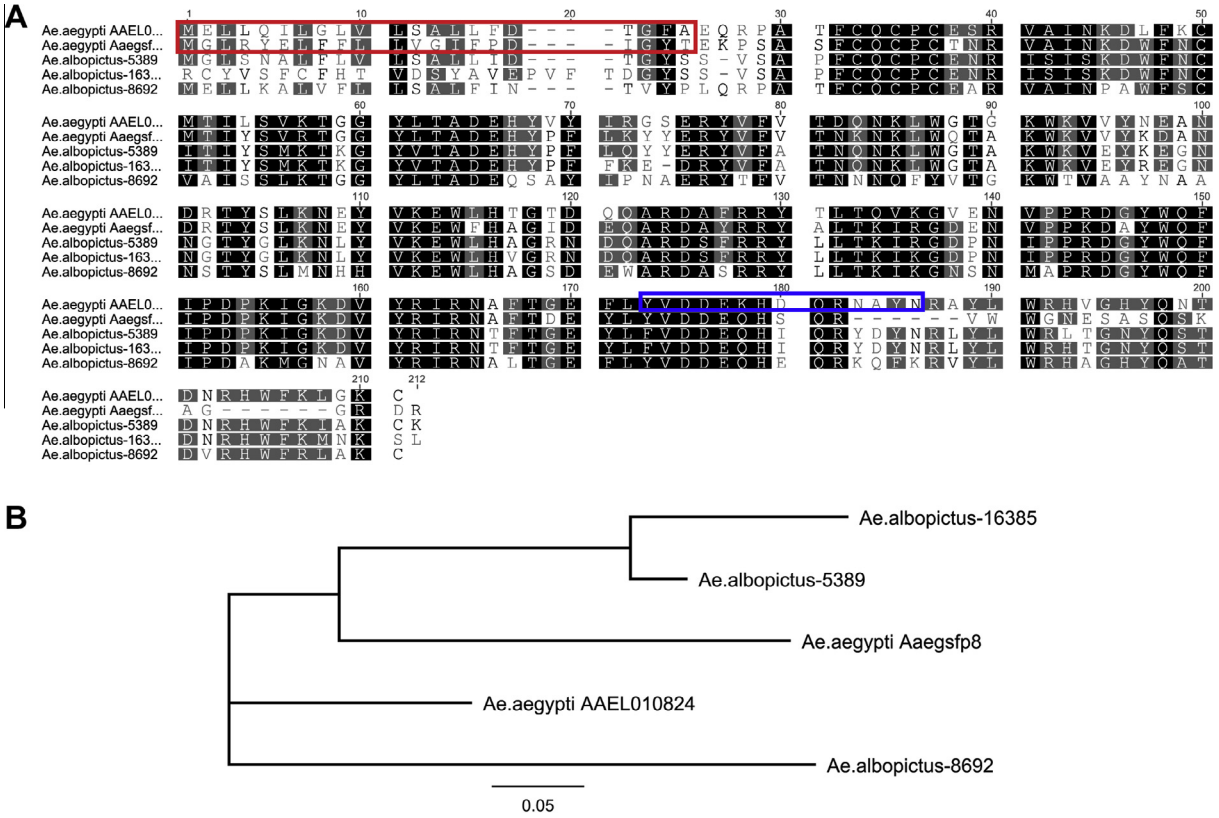

Fig. 1. Characterization of AAEL010824. (A) Multiple amino acid sequence alignment of AAEL010824 and AaegSfp8 from *A. aegypti* and 5389, 16385 and 8692 from *A. albopictus*. Identical amino acid residues in all sequences are shaded in black. Similar amino acids found in at least three of the sequences are shaded in grey. Predicted signal peptides in AAEL010824 and AaegSfp8 from *A. aegypti* and 5389 from *A. albopictus* are boxed in red. The peptide used for anti-AAEL010824 antibody production is boxed in blue. (B) Phylogenetic analysis derived by neighbor-joining analysis using *A. aegypti* AAEL010824 and Aaegsfp8 and *A. albopictus* 5389, 16385 and 8692 sequences. The bar represents a sequence divergence of 5%.

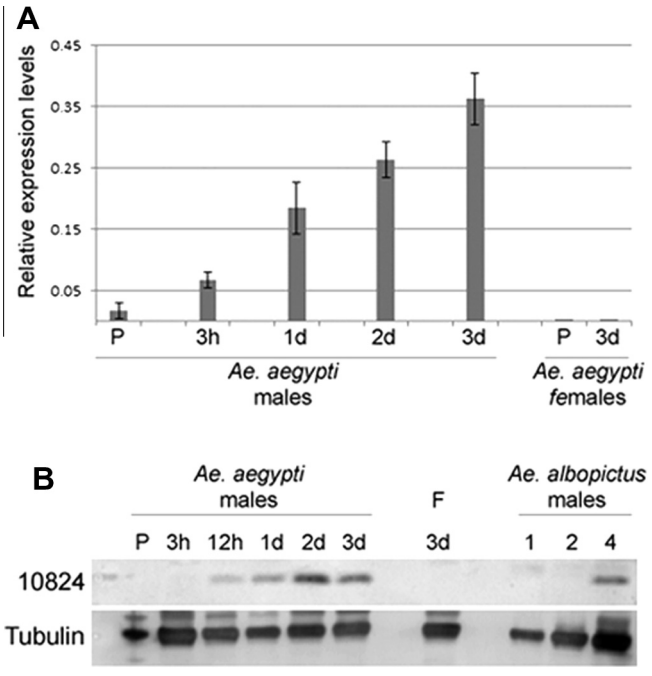

Fig. 2. *AAEL010824* expression. (A) Expression patterns of *AAEL010824* gene using quantitative PCR. Each sample was obtained from 3, late pupae (P), 3 h, 1, 2 or 3 day old males and 3 day old females. Each sample represents three different biological replicates. Relative expression values were calculated by normalizing the expression with RPS17. (B) Protein levels of AAEL010824 of an entire pupae or single reproductive tracts from virgin adults (males as indicated; F = females) were determined by Western blot. In addition, samples from 1, 2 and 4 reproductive tracts of *A. albopictus* males were tested.

support the hypothesis (Ramalingam, 1983) that increased AGs diameter during male adult maturation reflects accumulation of secreted Sfps. AAEL010824 was not detected in 3 day-old unmated females. Consistent with our phylogenetic data (above), we detected a signal with anti-AAEL010824 in protein samples from four *A. albopictus* AGs, suggesting the presence of an AAEL010824 ortholog in this species.

3.3. Expression driven by the AAEL010824 regulatory region is in the anterior cells of male AGs

AAEL010824 was previously described as a protein found in the male *A. aegypti* seminal fluid, that was encoded by a male-specific mRNA (Sirot et al., 2011, 2008). To confirm its AGs origin, we used anti-AAEL010824 to probe Western blots of proteins from specific tissue(s) of the male reproductive tract. AAEL010824 was detected in the AGs and not in the testes or the seminal vesicle (Fig. 3A).

Male *A. aegypti* AGs are composed of two distinct types of secretory cells, forming the anterior and posterior regions of the glands (Dapples et al., 1974). To determine if *AAEL010824* is expressed in the anterior cells of the AGs, the posterior cells, or both, we generated a transgenic line in which the expression of the fluorescent reporter, Enhanced Green Fluorescent Protein (EGFP), is driven by the *AAEL010824* regulatory region. Sequences comprising 5 kb of DNA upstream of the ATG that initiates translation of AAEL010824 and contain the apparent transcriptional start site, as identified by 5′ RACE, were cloned into pBAC[3xP3-EGFP-DsRedaf] (Fig. 3B). Confocal microscopy of AGs from transgenic males containing this construct revealed differential distribution of the EGFP signal: only cells in the anterior portion of the AGs showed a fluorescent signal (Fig. 3C); the signal was seen in the cytoplasm of those cells.

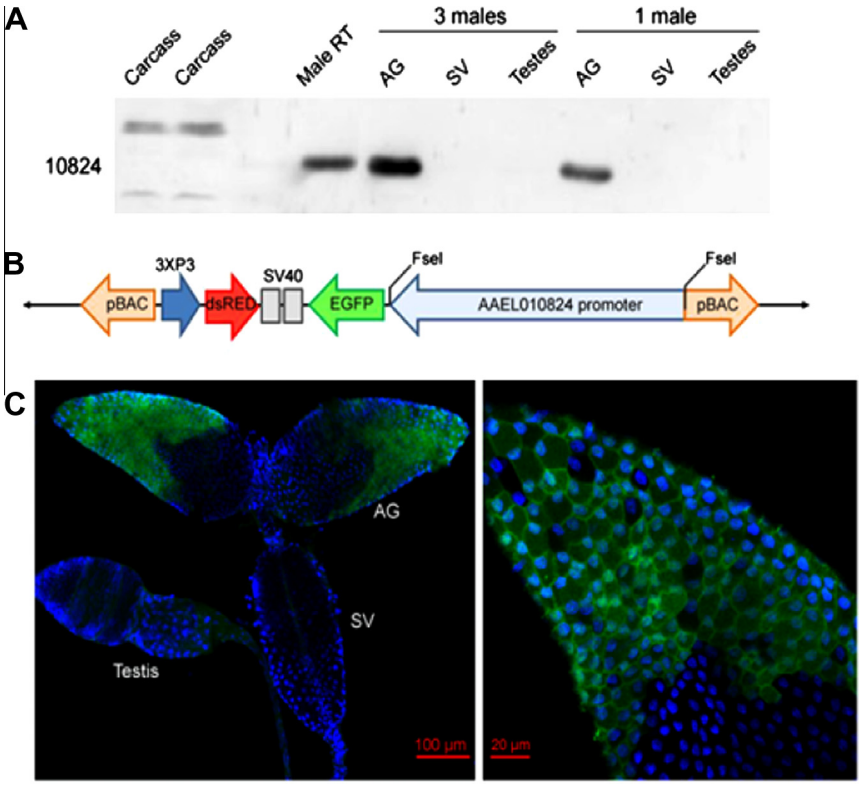

Fig. 3. Localization of AAEL010824 within the AGs. (A) AAEL010824 distribution in the male reproductive tract. Tissues tested included the carcass, AG, SV (seminal vesicle) and testes. (B) Overview of the final promoter construct: pBac[3xP3-AAEL010824EGFP-DSRedaf] (Not to scale). (C) Expression of EGFP under the control of the *AAEL010824* promoter is specific to the anterior cells of the AGs. Nuclei were stained blue using DAPI.

Expression of EGFP under the control of the *AAEL010824* regulatory region in the anterior region of the AGs is interesting, as secretions from these cells have been associated with inhibition of multiple inseminations, stimulation of oviposition (Ramalingam, 1983) and changes in host seeking behavior (Naccarati et al., 2012) in females. The *AAEL010824* regulatory region drives expression only in a portion of AG cells suggesting that the two cell-types might differ in the proteins they secrete or that they might have an essential, non-secretory role in reproduction. There is evidence that the two major cell-types, main and secondary cells, that constitute the *Drosophila melanogaster* AGs have unique functions, with the main cells products, such as ovulin and sex peptide, playing an important role in female post-mating responses (Kalb et al., 1993) and with the secondary cells (those at the distal end of the gland) potentially responsible for post-translational modification of some secreted AG proteins (Gligorov et al., 2013). To fully understand the role of the anterior and posterior cells of the *A. aegypti* AGs, it will be important to further identify and characterize the products of these cells to determine their individual roles in reproduction.

3.4. Depletion of *AAEL010824* in males and transfer to females

When the AGs mature, their lumens become filled with proteins secreted from the anterior and posterior cells. Since *Aedes* males are polygynous, at each copulation, some of AGs luminal content is transferred to females during ejaculation. After transfer, secretions must then be replenished by the secretory cells of the AGs. In *Drosophila*, main cells of the AGs increase gene expression after mating, presumably to replenish secreted proteins transferred at mating. However, gene expression in the secondary cells of the AGs remains stable, regardless of male age or sexual activity (Bertram et al., 1992; DiBenedetto et al., 1990). We used *AAEL010824* as a marker to determine: (1) how levels of the gene change after mating, (2) how many sequential matings are required to deplete AAEL010824, and (3) how long it takes for the AGs to replenish their complement of AAEL010824.

To answer the first question, we used qPCR to measure *AAEL010824* mRNA levels from virgin males and males mated successively to 1, 2 or 3 females at 0, 24 and 48 h after copulation. Levels of *AAEL010824* mRNA in virgin males remained stable. However,

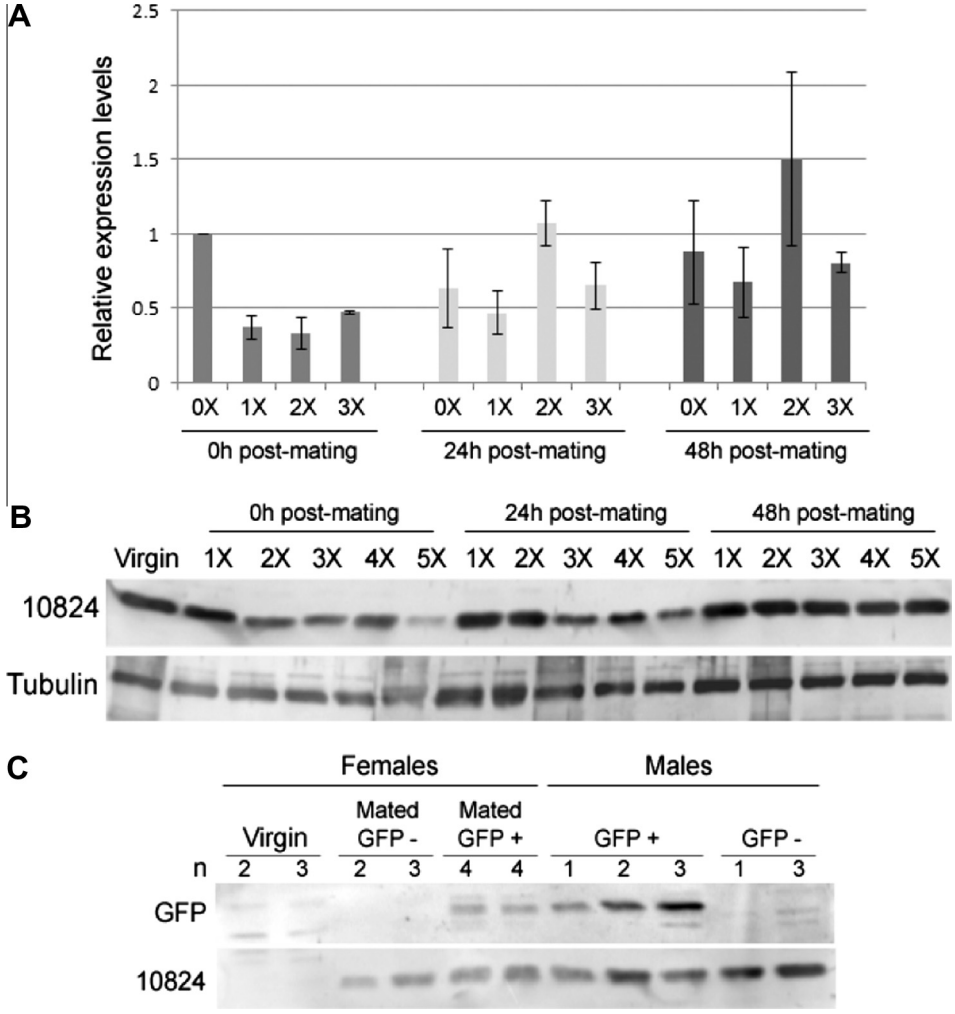

Fig. 4. Transfer, depletion and replenishment of AAEL010824. (A) mRNA levels of *AAEL010824* using quantitative PCR. Samples were obtained from virgin males and males mated to 1, 2 and 3 females successively (1×, 2×, 3×). Samples were collected at 0, 24 and 48 h after mating. Each sample represents four different biological replicates. Relative expression values were calculated by normalizing the expression with RPS17. Columns followed by the same letter are not significantly different from each other within age group (Univariate analysis: $F = 7.06$, df = 3, $P = 0.001$). Values across time classes are significantly different (ANOVA, df = 14, $F = 4.6$, $P = 0.03$; LSD separation of means, 0 h is different from 24 h ($P = 0.02$), 0 h is different from 48 h ($P = 0.03$), no significant difference between 24 and 48 h ($P = 0.91$)) (B) Protein levels of AAEL010824 were analyzed by Western blotting. Protein samples were extracted from single reproductive tracts of virgin males and males mated to 1,2,3,4 and 5 females. Samples were collected 0, 24 and 48 h post-mating. The figure shows a representative blot from one of three replicates of this experiment. Anti-tubulin was use as loading control. (C) EGFP transfer from male to females was analyzed by Western blotting. Proteins were extracted from reproductive tracts from males expressing EGFP under the *AAEL010824* promoter (GFP+), wild-type males (GFP−), from virgin females and females mated to EGFP males and wild-type males. Anti-AAEL010824 was used as control for Sfp transfer.

immediately after copulation, *AAEL010824* mRNA levels of mated males (mated 1, 2 or 3 times) decreased around 2-fold relative to the levels in virgins. *AAEL010824* mRNA levels of mated males then generally increased with time, reaching similar levels to those of virgin males at 48 h post-mating, indicating high levels of mating-induced gene expression (Fig. 4A). For reasons that are unclear, males that are mated twice have higher levels of AAEL010824 mRNA after 24 and 48 h. However, this may be an effect of natural variation in transcript levels or possibly result from the sensitivity of qPCR in detecting differences in sample preparation.

We used Western blotting and Image Studi Lite software to examine AAEL010824 levels after 1, 2, 3, 4 or 5 successive matings. AGs of males were depleted of AAEL010824 by approximately 44% after 2 matings and 72% after mating with 5 females within a 5 h period. This result is consistent with previous studies that showed that after 4–5 consecutive matings, males were depleted of factors that affected fecundity and longevity of subsequent female mates (Helinski and Harrington, 2011). We next examined the replenishment of AAEL010824 in 5×-mated males that were allowed to recover (in the absence of females) for 0 h, 24 h and 48 h. We observed that virgin-levels of AAEL010824 in AGs were replenished by 48 h of recovery (Fig. 4B). These findings—consistent with previous work showing AG size in sequentially mated mosquitoes was reduced by half following multiple matings, returning to their normal size after a rest period of 3 days (Dapples et al., 1974; Foster and Lea, 1975)—further refine our knowledge of the reproductive ability of *Aedes* males and potentially give insight into determining the number of males required, and the timing of their release, in genetic mosquito control programs.

We next tested for transfer of EGFP to females during copulation. Previous studies of the anterior AG cells suggested that they used apocrine secretion mechanisms, in which the cytoplasm pinches off and falls freely into the lumen of the gland (Dapples et al., 1974; Ramalingam, 1983). Since the EGFP in our construct lacks a predicted signal sequence, we used our *AAEL010824* promoter-EGFP line to test whether this mechanism of secretion might be operating in the anterior cells. Virgin females were mated with *AAEL010824* promoter-EGFP males. The females' reproductive tracts were dissected immediately post mating, and probed for EGFP on Western blots, using anti-GFP. As a control, anti-AAEL010824 was used to detect transfer of protein from male to female (Fig. 4C). We detected EGFP in reproductive tract extracts from the mated females (Fig. 4C). Our observation of EGFP in the females' reproductive tracts is consistent with apocrine secretion from the anterior cells. However, we cannot rule out that the EGFP derived from its release by dying or lysing cells, or that whole cells (containing EGFP) were transferred to the females, in a mechanism recently reported for *Drosophila* AG secondary cells (Leiblich et al., 2012).

4. Conclusions

Previous studies indicated that male AGs play an important role in the reproductive success of mosquitoes (reviewed in Sirot et al. (2011)). AG secretions, including Sfps, are transferred to females during mating. Sfp receipt by the female results in significant physiological and behavioral changes which include a decrease in mating probability (Fuchs et al., 1968; Helinski and Harrington, 2011) and an increase in blood feeding behavior (Lee and Klowden, 1999). Although the nature and sequences of these proteins have been investigated in other species, the structure and regulation of the proteins in the male mosquito AGs have received little attention to date. Our results provide new information on the function of these important reproductive organs in male mosquitoes.

Here, we characterized the *A. aegypti* male AGs, using AAEL010824, or EGFP fused to its gene-regulatory region, as a marker. We previously identified this Sfp as a male-derived protein that is transferred to females during mating (Sirot et al., 2011). We show here that AAEL010824 is found exclusively in the AGs of the male reproductive tract. Our AAEL010824 expression data shows that this AGs protein expressed after eclosion, with its levels increasing for at least 3d. In addition, we showed that a male's complement of AAEL010824 is largely depleted after mating with 5 consecutive females in succession, and that 48 h of recovery suffice for it to be fully replenished. If AAEL010824 is representative of AG proteins, then these results demonstrate the limits of depletion and replenishment of AG secretions in males and will ultimately guide functional studies of AG proteins with RNAi. Finally, we generated a transgenic line in which EGFP is driven by the *AAEL010824* regulatory region. Using this line, we showed that the *AAEL010824* regulatory region drives expression exclusively in the anterior cells of the AGs. Surprisingly, we observed transfer of EGFP (which lacks a signal sequence) from male to females during copulation, suggesting an apocrine mechanism for secretion. However, other processes can be involved in protein transfer during mating. Because of the tissue-specificity of our transgenic line, we suggest that the *AAEL010824* regulatory region can be used to express other proteins into the male AGs. Our results on the function and secretion pattern of the male AGs in mosquitoes, coupled with our identification of an AG-specific regulatory region, will enable future studies of mosquito mating biology and thus will significantly advance the study of reproductive targets for vector control.

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